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(57) Abstract

The present invention provides a nuclease resistant single-stranded non-naturally occurring nucleic acid molecule having a formula: $[(D)x_a(R)y_a]_a[(D)x_{b-y}(R)y_{b-y}]_{b-y}$ $[(D)x_z(R)y_z]_z$, wherein each D represents a deoxyribonucleotide which may be the same as, or different from, any other deoxyribonucleotide present in the molecule; wherein each R represents a ribonucleotide which may be the same as, or different from, any other ribonucleotide present in the molecule; wherein each of x_a, y_a x_{b-y}, y_{b-y}, x_z and y_z represents an integer which may independently vary from 0 to 10; and wherein each of a, b-y, and z represents an integer which may independently vary from 0 to 50.

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NUCLEASE RESISTANT, SINGLE-STRANDED, NON-NATURALLY OCCURRING NUCLEIC ACID MOLECULES

BACKGROUND OF THE INVENTION

Nucleic acid probes of DNA or RNA are known to be useful for detecting complementary sequences in the presence of a large amount of non-complementary DNA or RNA. However, one problem encountered in the use of such probes is their sensitivity to enzymatic, particularly, nuclease degradation.

Further, the use of antisense RNA as a therapeutic agent has been suggested. However, susceptibility to enzymatic, i.e. nuclease degradation, impedes such an approach.

European Patent Publication No. 067,597, published December 22, 1982, discloses oligonucleotides and a process for their preparation which comprises incorporating ribonucleotide units at specific locations in deoxyribonucleotide chains thus providing predetermined cleavage sites which allow ease of chain cleavage.

Coassigned U.S. Serial No. 805,279,filed December 5, 1985, now allowed discloses synthetic, non-naturally occurring molecules represented by the formula

$$[NA_1---S---NA_2]_n$$

wherein NA_1 and NA_2 are different noncomplementary nucleic acid sequences; wherein ---S--- is a scissile linkage which is capable of being cleaved or disrupted without cleaving or disrupting the nucleic acid sequences of NA_1 or NA_2 or of a target nucleic acid

sequence capable of hybridizing to said composition, wherein if the scissile linkage is a nucleic acid sequence it is RNA when both NA_1 and NA_2 are DNA sequences, or the scissile linkage is DNA when both NA_1 and NA_2 are RNA sequences; and wherein n is an integer from 1 to 4.

Coassigned U.S. Serial No. 187,814, filed April 29, 1988 as a continuation-in-part of U.S. Serial No. 805,279, discloses a method for detecting a target nucleic acid molecule using the molecules disclosed in U.S. Serial No. 187,814.

U.S. Patent No. 4,359,535, issued November 16, 1982 discloses autonomously replicating DNA containing inserted DNA sequences.

U.S. Patent No. 4,563,417, issued January 7, 1986 discloses nucleic acid hybridization assays employing antibodies to intercalation complexes.

Melton et al., Nucleic Acids Research, Vol. 12, No. 18 (1984) discloses in vitro synthesis of biologically active RNA and RNA hybridized probes.

The present invention relates to novel nucleic acid molecules which possess enhanced resistance to nuclease degradation and thus are particularly advantageous when used either as probes or in anti-sense or other therapeutic applications.

SUMMARY OF THE INVENTION

The present invention provides a nuclease resistant single-stranded non-naturally occurring nucleic acid molecule having the formula:

$$[(D)x_a(R)y_a]_a\ [(D)x_{b-y}(R)y_{b-y}]_{b-y}\ \ [(D)x_z\ (R)y_z]_z$$

wherein each D represents a deoxyribonucleotide which may be the same as, or different from, any other deoxyribonucleotide present in the molecule; wherein each R represents a ribonucleotide which may be the same as, or different from, any other ribonucleotide present in the molecule; wherein each of x_a, y_a, x_{b-y}, y_{b-y}, x_z and y_z represents an integer which may independently vary from 0 to 10; and wherein each of a, b-y, and z represents an integer which may independently vary from 0 to 50.

The invention also concerns a method of inhibiting the proliferation of tumor cells which comprises contacting the tumor cells with a growth inhibiting amount of a nuclease resistant, single-stranded, non-naturally occurring, nucleic acid molecule having a nucleic acid sequence hybridizable with the mRNA transcript of a gene of the tumor cells essential for proliferation thereof having the formula:

$$[(D)x_a(R)y_a]_a[(D)x_{b-y}(R)y_{b-y}]_{b-y}\ \ [(D)x_z\ (R)y_z]_z$$

under suitable conditions so as to inhibit proliferation of tumor cells.

Finally, a method of inhibiting the replication of a virus is provided. The method which comprises

contacting the virus with a replication inhibiting amount of a nuclease resistant single-stranded non-naturally occurring molecule having a nucleic acid sequence complementary to the nucleic acid sequence of the virus and comprising the structure:

$$[(D)x_a(R)y_a]_a[(D)x_{b-y}(R)y_{b-y}]_{b-y}\ \ [(D)x_z\ (R)y_z]_z$$

under suitable conditions so as to inhibit replication of the virus.

BRIEF DESCRIPTION OF FIGURES

Figure 1 depicts polymer-supported DNA synthesis on silica gel or CPG supports.

Figure 2 depicts polymer-supported RNA synthesis on silica gel or CPG supports.

Figure 3 depicts the synthesis of DRDR sequences first attaching a nucleoside onto an insoluble support and then placing the support in a small column and attaching the column to an automated DNA/RNA synthesizer.

Figure 4 Blots of DRDR Sequences Showing Increased Nuclease Resistance With 2′ Protection

Figure 5 Blots of DRDR Sequences Showing Increased Nuclease Resistance With 2′ Protection

Figure 6 Blots of DRDR Sequences Showing Increased Nuclease Resistance With 2′ Protection

DETAILED DESCRIPTION OF THE INVENTION

The present invention provides a nuclease resistant, single-stranded, non-naturally occurring nucleic acid molecule having the formula:

$$[(D)x_a(R)y_a]_a[(D)x_{b-y}(R)y_{b-y}]_{b-y}\ [(D)x_z\ (R)y_z]_z$$

wherein each D represents a deoxyribonucleotide which may be the same as, or different from, any other deoxyribonucleotide present in the molecule; wherein each R represents a ribonucleotide which may be the same as, or different from, any other ribonucleotide present in the molecule; wherein each of x_a, y_a, x_{b-y}, y_{b-y}, x_z and y_z represents an integer which may independently vary from 0 to 10; and wherein each of a, b-y, and z represent an integer which may independently vary from 0 to 50.

In a presently preferred embodiment, each of x_a, y_a, x_{b-y}, y_{b-y}, x_z and y_z independently varies and is less than or equal to 5. Additionally, each of a, b-y, and z independently varies and is less than or equal to 25.

Further, each D is selected from the group consisting of deoxyadenylate, deoxyguanylate, deoxythymidylate, deoxycytidylate and analogs or derivatives thereof. Moreover, each R is selected from the group consisting of adenylate, guanylate, uridylate, cytidylate and analogs or derivatives thereof.

It will be clear to one skilled in the art that the deoxyribonucleotides and ribonucleotides useful in this invention encompass all deoxyribonucleotides and ribonucleotides useful in the practice of this

invention disclosed herein.

In one embodiment, the nucleic acid molecules of the invention have at least one R to the 2′ position of which a protecting group, e.g. a silyl or a lower alkyl (C_1-C_5) group, which enhances the resistance of the molecule to digestion by a nuclease, is attached.

Additionally or alternatively, the nuclease resistant, single-stranded, non-naturally occurring nucleic acid molecules of this invention has at least one D or R which comprises a hydrogen atom in place of a hydroxyl group on the phosphorus atom.

The invention also provides the nuclease resistant, single-stranded, non-naturally occurring nucleic acid molecules hybridizable with a messenger RNA transcript of a viral gene, e.g. an HIV gene which encodes a gene product essential for viral, e.g. HIV replication.

Alternatively, the nuclease resistant, single-stranded, non-naturally occurring nucleic acid molecule are hybridizable with a regulatory sequence of a viral, e.g. an HIV gene essential for viral replication.

The invention also provides nuclease resistant, single-stranded, non-naturally occurring nucleic acid molecules hybridizable with a messenger RNA transcript of a Coxsackie B-3 gene which encodes a gene product essential for Coxsackie B-3 replication.

Alternatively, the nuclease resistant, single-stranded, non-naturally occurring nucleic acid molecules are hybridizable with a regulatory sequence of a Coxsackie B-3 gene essential for Coxsackie B-3

replication.

This invention also provides a method of inhibiting the replication of the HIV virus which comprises contacting the a regulatory sequence of an HIV gene essential for replication with a nucleic acid molecule of this invention so as to form a complex therewith and thereby inhibit the replication of the HIV virus.

The invention further provides a method of inhibiting the replication of the HIV virus which comprises contacting a messenger RNA transcript of an HIV gene essential for replication with a nucleic acid molecule of this invention so as to form a complex therewith and thereby inhibiting the proliferation of the HIV virus.

Additionally, the invention provides a method of inhibiting the replication of the Coxsackie B-3 virus which comprises contacting a messenger RNA transcript of a Coxsackie gene essential for its replicationn with a nucleic acid molecule of this invention so as to form a complex therewith and thereby inhibit replication of the Coxsackie B-3 virus.

Still further, the invention provides a method of inhibiting the proliferation of the Coxsackie B-3 virus which comprises contacting the molecule with a regulatory sequence of a Coxsackie B-3 gene essential for its replication with a nucleic acid molecule of this invention so as to form a complex therewith and thereby inhibit replication of the Coxsackie B-3 virus.

The invention also concerns a method of inhibiting the proliferation of tumor cells which comprises contacting the tumor cells with a proliferation inhibiting amount

of a nuclease resistant, single-stranded non-naturally occurring, nucleic acid molecule having a nucleic acid sequence hybridizable with an mRNA transcript of a gene of the tumor cells essential for proliferation thereof, the molecule having the formula:

$$[(D)x_a(R)y_a]_a[(D)x_{b-y}(R)y_{b-y}]_{b-y} \quad [(D)x_z \ (R)y_z]_z$$

under suitable conditions so as to inhibit the proliferation of the tumor cells.

Additionally, the invention concerns a method of inhibiting the replication of a virus which comprises contacting the virus with a replication inhibiting amount of a nuclease resistant single-stranded non-naturally occurring molecule having a nucleic acid sequence hybridizable to the nucleic acid sequence of the virus and having the formula:

$$[(D)x_a(R)y_a]_a[(D)x_{b-y}(R)y_{b-y}]_{b-y} \quad [(D)x_z \ (R)y_z]_z$$

and under suitable conditions so as to inhibit the replication of the virus.

This invention still further provides a method of treating a subject afflicted with a disorder which comprises administering, e.g. by intravenous administration or by time release implant, to the subject an effective amount of a nuclease resistant, single-stranded non-naturally occurring molecule of this invention alone or in and a pharmaceutically acceptable carrier, the moelcule being characterized by its ability to interfere at the nucleic acid level with the progression of, or symptoms associated with, the disorder.

This invention is illustrated in the Experimental Details section which follows. This section is set forth to aid in an understanding of the invention but is not intended to, and should not be construed to, limit in any way the invention as set forth in the claims which follow.

EXPERIMENTAL DETAILS

MATERIALS AND METHODS

POLYMER-SUPPORTED DNA-RNA SYNTHESIS

Synthesis of DNA (Figure 1). Table I shows the protocol for synthesis of DNA on silica gel or CPG supports. The synthesis was completed on Vydak Silica gel or CPG LCAA using 2-5 micromoles of nucleoside on the support. The synthesis was accomplished using an automated synthesizer (1) with a continuous flow manner (3.0-3.5 ml/min), and the reactions were carried out in a column. Condensations were completed under dry nitrogen, and phosphoramidites of the four bases were kept under nitrogen. At the completion of the synthesis, oligonucleotide cleavage and deprotection were effected by treatment with: 1) Dioxane: Triethylamine: thiophenol (2:1:1 by volume) at room temperature for 1 hour (only for OMe derivatives); and 2) concentrated ammonia at 55°C for 24 hours. The oligonucleotides were purified from the resulting mixture by thin layer chromatography (TLC) on silica gel (Kieselgel 60 plates, Merck) with a mixture of n-propanol: ammonia: water (55:35:10) as the running solvent. The purity and size of the final products were confirmed by electrophoretic analysis on polyacrylamide gels.

Synthesis of RNA (Figure 2). Table II shows the protocol for synthesis of RNA on silica gel or CPG supports. The synthesis was completed on fractosil, Vydak, or CPG, using 2-5 micromoles of nucleoside on the support. Reactions were carried out in a column using an automated synthesizer with a continuous flow

manner (3.0-3.5 ml/min) (G. Alvarado-Urbina et al. (1986) Biochem. Cell Biol. 64:548-555). At the completion of synthesis, oligonucleotide cleavage and deprotection were effected by treatment with: 1) Dioxanne: triethylamine: thiophenol (2:1:1 by volume) at room temperature for 1 hour; and 2) concentrated ammonia: ethanol (3:1) for 24 hours at room temperature and 16 hours at 50°C. The crude material was then treated with TBAF ($[CH_3(CH_2)_3]_4NF$-tetrabutylammonium fluoride, 1.0 M) (1 ml) for 6 hours at room temperature (E.J. Corey and Barry B. Snider (1972) J. Am. Chem. Soc. 94:2549). Excess TBAF was converted to NaF with Na^+ ion exchange resin and the nucleotide desalted (Sephadex $^G50$). The completely deprotected material was obtained by purification on TLC (silica gel) or by polyacrylamide gel electrophoresis.

Table I

Protocol for synthesis of DNA on silica gel or CPG supports (2-5 micromole scale)

Step	Reagent or Solvent Mixture	Time (min:sec)		Vol (ml)
1	Dichloroethane	1:30	5.0	
2	3% DCA in dichloroethane	1:30	5.0	
3	Dichloroethane	1:00	3.5	
4	Acetonitrile	2:00	5.0	
5	Condensation-Phosphoramidite In line mixing - 20 micromoles of phosphoramidite/CH_3CN and 3% Tetrazol in CH_3CN	1:00	3.5	
	Recycling	1:00	--	
6	Oxidation - 0.01 M Iodine in a mixture of CH_3CN:H_2O:2-lutidine	0:20	1.0	

7	Capping (in line mixing) a) 20% $(AcO)_2$ in CH_3CN: 2-Glutidine (80:20) b) 3% DMAP in CH_3CN	1:00	3.5	xxxx
	Recycling	1:00	--	x
	Total Time	10:20		

Table II

Protocol for Synthesis of RNA on Silica or CPG Supports (2-5 micromoles of nucleoside)

Step	Reagent or solvent mixture	Time (min:sec)
1	Dichloroethane	1:30
2	3% DCA in Dichloroethane	1:30
3	Dichloroethane	1:00
4	Acetonitrile	2:00
5	Condensation 50:50 In line mixing -50 micromoles of the phosph-oramidite and 3% Tetrazole in acetonitrile	1:00
	Recycling	5:00
6	Acetonitrile	0:30
7	0.01M I_2 in CH_3CN:H_2O:2-lutidine (120:120:24)	0:20
8	In line mixing DMAP-CH_3CN (3%) and 10% $(AcO)_2$ in CH_3CN:2 lutidine (80:20)	1:00
	Recycling Go to step #1	1:00

PREPARATION OF DRDR... SEQUENCES USING THE POLYMER SUPPORTED APPROACH

Sequences. Examples of the sequences involved (5′-3′) are shown below.

1) $CaAcCcAcAgGcTgTuTuAa_3$,
2) $CgCaCcGaAuGcGgAgTuTu_3$,

The Reaction. The preparation of DRDR... sequences is shown in Figure 3. For the preparation of mixed sequences DNA-RNA-DNA-RNA...$_n$, applicants' general strategy was to attach the 3′-terminal nucleoside of an intended sequence onto the insoluble support. Once the first nucleoside has been attached, the support is used as substrate for chain extension reactions. Applicants used mainly Vydak silica gel with an amount of loading equal to 65-80 micromoles/gram, or long chain alkyl amine controlled pore glass (LCAA CPG) with a loading of 25-30 micromoles/gram. The polymer support with the first nucleoside attached to it was placed in a small column and attached to an automated DNA/RNA synthesizer.

The synthesis cycle employed was described above for DNA and RNA separately. For the preparation of DRDR... sequences, the synthesis cycles are combined into four basic steps: 1) acidic treatment to remove 5′-dimethoxytrilyl protecting groups; 2) condensation of the polymer bound nucleoside with a nucleoside-3′-diisopropylphosphoramidite for DNA and RNA; 3) oxidation, using iodine and water in acetonitrile, to convert the phosphite linkage into phosphate linkage; and 4) capping with acetic anhydride and DMAP to block off unreacted sites and to remove residual moisture.

The most difficult step is the chain extension reaction. Unlike 2'-deoxyribonucleosides, protected ribonucleosides are much more hindered about the 3' position. This is due to the bulky t-butyl dimethylsilyl or triisopropylsilyl protecting groups which applicants use on the 2' position. Consequently, applicants have found it necessary to use higher concentrations (50 micromoles vs 20 micromoles) and longer coupling times (6 minutes vs 2 minutes) relative to phosphoramidite-OMe of oligodeoxyribonucleotide synthesis in order to obtain reasonable coupling yields.

Deprotection and Purification of the Final Product. After assembly of the desired sequence by the automated synthesizer, the final product - a mixed sequence DNA-RNA-DNA...$_n$ - must be cleaved from the polymer, deprotected and isolated. This procedure is more difficult in the case of RNA sequences than for DNA sequences for two reasons. First, the 2'-protecting group requires the inclusion of an extra deprotection step. Second, the deprotected oligoribonucleotides are much more sensitive toward chemical hydrolysis.

The deprotection began by treatment of the polymer support with thiophenoxide-Dioxane: TEA:Ph5H (2:1:1 by volume) at room temperature for 1 hour. This is to remove the methyl protecting groups. Acyl linkages were then hydrolyzed by treatment with ethanolic ammonium hydroxide (NH_4OH:EtOH, 3:1) at room temperature for 72 hours. The crude material was finally desilylated with a TBAF solution, desalted and purified on TLC or polyacrylamide gel electrophoresis.

All of the above mixed sequences were characterized by kinasing samples and sizing them.

EXAMPLES

EXAMPLE 1

Applicants have created many nuclease resistant, single stranded, non-naturally occurring nucleic acid molecules by the polymer supported approach discussed in Materials and Methods. Table III illustrates the increased nuclease resistance of 5 of these sequences. RNase cleaves single-stranded RNA, DNase cleaves single stranded DNA and S1 breaks down both DNA and RNA. Table III also illustrates the effect of 2′ protection of the ribonucleotide by a silyl group.

Although the Materials are more resistant to some nucleases than DNA or RNA alone, the addition of a 2′ protecting group, in some instances, confers even greater nuclease resistance.

Table III

Nuclease Resistant Constructions

No. 136	DRDRDRDRDRDRD	13-mer
No. 148	DDDDRDDDDRDDDDRDDDD	19-mer
No. 149	DDDRDDDRDDDRDDDRDDD	19-mer
No. 150	DDRDDRDDRDDRDDRDD	17-mer
No. 137	RRRRRRRRRRRR	12-mer

	Si+		Si-	
	RNase	S1	RNase	S1
No. 137(R)	+	-	-	-
No. 136(DR)	+++	++	-	-
No. 150(DDR)	+++	++	+(+)	-
No. 149 (DDDR)	+++	++	+	-
No. 148(DDDDR)	++(+)	++	+	-

+++ Very resistant
++ Moderately resistant
+ Slightly resistant
- Not resistant

No. 082	GTTGTGTAGACTCACTCGTGAACCTAGATT3'
No. 136	TuTuCcCaGuCaC3'
No. 137	auucacacaacc3'
No. 148	GGTTuTCCCaGTCAcGACG3'
No. 149	TTTucCCaGTCaCGAcGTT3'
No. 150	TTcCCaGTcACgACgTT3'

EXAMPLE 2

The nucleic acid molecule of the present invention are useful as nucleic acid probes because of their resistance to nuclease degradation. Preferably, the 2' position of one or more ribonucleotides in the molecule may be substituted with a protecting group which enhances the nuclease resistance of the resulting molecule.

EXAMPLE 3

The nucleic acid molecules of the present invention may be used to deliver therapeutic agents into contact with DNA or RNA targets and are particularly advantageous for this purpose because of their resistance to nuclease degradation. Preferably, the 2′ position of one or more ribonucleotides within the molecule may be substituted with a protecting group which enhances the nuclease resistance of the resulting copolymer. More than one such protecting group may be present in the molecule, each such group being the same as, or different from one or more other such groups. Further, the therapeutic agent may be attached to the molecule through any available reactive site on the molecule, one such site being a 2′ position on a ribonucleotide which has not had a protective group placed thereon.

EXAMPLE 4

The constructions of nuclease resistant single-stranded nucleic acid molecules, because of their enzymatic resistance, can also be of use for antisense blocking or otherwise modifying the transcription and/or translation of nucleic acid strings by way of competitive inhibition.

Applicants have been able to show that nuclease resistant single-stranded nucleic acid molecules are effective in inhibiting the replication of Coxsackie B-3 virus. The experimental data for this is the reduction of the number of viral plaques in an activity assay in which a known titer of virus is exposed to a cell culture. The control develops plaques indicating viral titer. The experimental treatment shows both a reduction in the number and the size of the viral plaques. Compared to DNA sequences, the DRDR type compounds are more stable and provide longer lasting inhibition.

EXAMPLE 5

Applicants have also shown that the molecules can be used to inhibit HIV (AIDS virus) replication. The molecule is complementary to an HIV gene which encodes a gene product essential for HIV replication. The molecule may also be complementary to an HIV gene which encodes the HIV T helper cell receptor.

What is claimed is:

1. A nuclease resistant, single-stranded, non-naturally occurring nucleic acid molecule having the formula:

$[(D)x_a(R)y_a]_a[(D)x_{b-y}(R)y_{b-y}]_{b-y}$ $[(D)x_z(R)y_z]_z$

wherein each D represents a deoxyribonucleotide which may be the same as, or different from, any other deoxyribonucleotide present in the molecule;

wherein each R represents a ribonucleotide which may be the same as, or different from, any other ribonucleotide present in the molecule;

wherein each of x_a, y_a, x_{b-y}, y_{b-y}, x_z and y_z represents an integer which may independently vary from 0 to 10;

Wherein each of a, b-y, and z represent an integer which may independently vary from 0 to 50.

2. A molecule of claim 1, wherein each of x_a, y_a, x_{b-y}, y_{b-y}, x_z and y_z independently varies and is less than or equal to 5.

3. A molecule of claim 1, wherein each of a, b-y, and z independently varies and is less than or equal to 25.

4. A molecule of claim 1, wherein each D is selected from the group consisting of deoxyadenylate, deoxyguanylate, deoxythymidylate, deoxycytidylate and analogs or derivatives thereof.

5. A molecule of claim 1, wherein each R is selected from the group consisting of adenylate, guanylate, uridylate, cytidylate and analogs or derivatives thereof.

6. A molecule of claim 5 wherein at least one R has attached to its 2' position a protecting group which enhances the resistance of the molecule to digestion by a nuclease.

7. A molecule of claim 6, wherein the protecting group is a silyl group.

8. A molecule of claim 6, wherein the protecting group is a lower alkyl group.

9. A molecule of claim 1, wherein at least one D or R comprises a hydrogen atom in place of a hydroxyl group on the phosphorus atom.

10. A molecule of claim 1, hybridizable with a messenger RNA transcript of an HIV gene which encodes a gene product essential for HIV replication.

11. A molecule of claim 1, hybridizable with a regulatory sequence of an HIV gene essential for HIV replication.

12. A molecule of claim 1, hybridizable with a messenger RNA transcript of a Coxsackie gene which encodes a gene product essential for Coxsackie B-3 replication.

13. A molecule of claim 1, hybridizable with a regulatory sequence of a Coxsackie B-3 gene essential for Coxsackie B-3 replication.

14. A method of inhibiting the proliferation of the HIV virus which comprises contacting the molecule of claim 1 with a messenger RNA transcript of an HIV gene so as to form a complex thereby inhibiting the proliferation of the HIV virus.

15. A method of inhibiting the proliferation of the HIV virus which comprises contacting the molecule of claim 1 with a messenger RNA transcript of an HIV gene so as to form a complex thereby inhibiting the proliferation of the HIV virus.

16. A method of inhibiting the proliferation of the Coxsackie B-3 virus which comprises contacting the molecule of claim 1 with a messenger RNA transcript of a Coxsackie gene so as to form a complex thereby inhibiting the proliferation of the Coxsackie B-3 virus.

17. A method of inhibiting the proliferation of the Coxsackie B-3 virus which comprises contacting the molecule of claim 1 with a regulatory sequence of a Coxsackie B-3 gene so as to form

a complex thereby inhibiting the proliferation of the Coxsackie B-3 virus.

18. A method of inhibiting the proliferation of tumor cells which comprises contacting the tumor cells with a growth inhibiting amount of a nuclease resistant, single-stranded non-naturally occurring, nucleic acid molecule having a nucleic acid sequence hybridizable with the mRNA transcript of a gene of the tumor cells essential for growth thereof having the formula:

$$[(D)x_a(R)y_a[(D)x_{b-y}(R)y_{b-y}]_{b-y}\ \ [(D)x_z\ (R)y_z]_z$$

under suitable conditions so as to inhibit the proliferation of the tumor cells.

19. A method of inhibiting the replication of a virus which comprises contacting the virus with a replication inhibiting amount of a nuclease resistant single-stranded non-naturally occurring molecule having a nucleic acid sequence complementary to the nucleic acid sequence of the virus and comprising the formula:

$$[(D)x_a(R)y_a[(D)x_{b-y}(R)y_{b-y}]_{b-y}\ \ [(D)x_z\ (R)y_z]_z$$

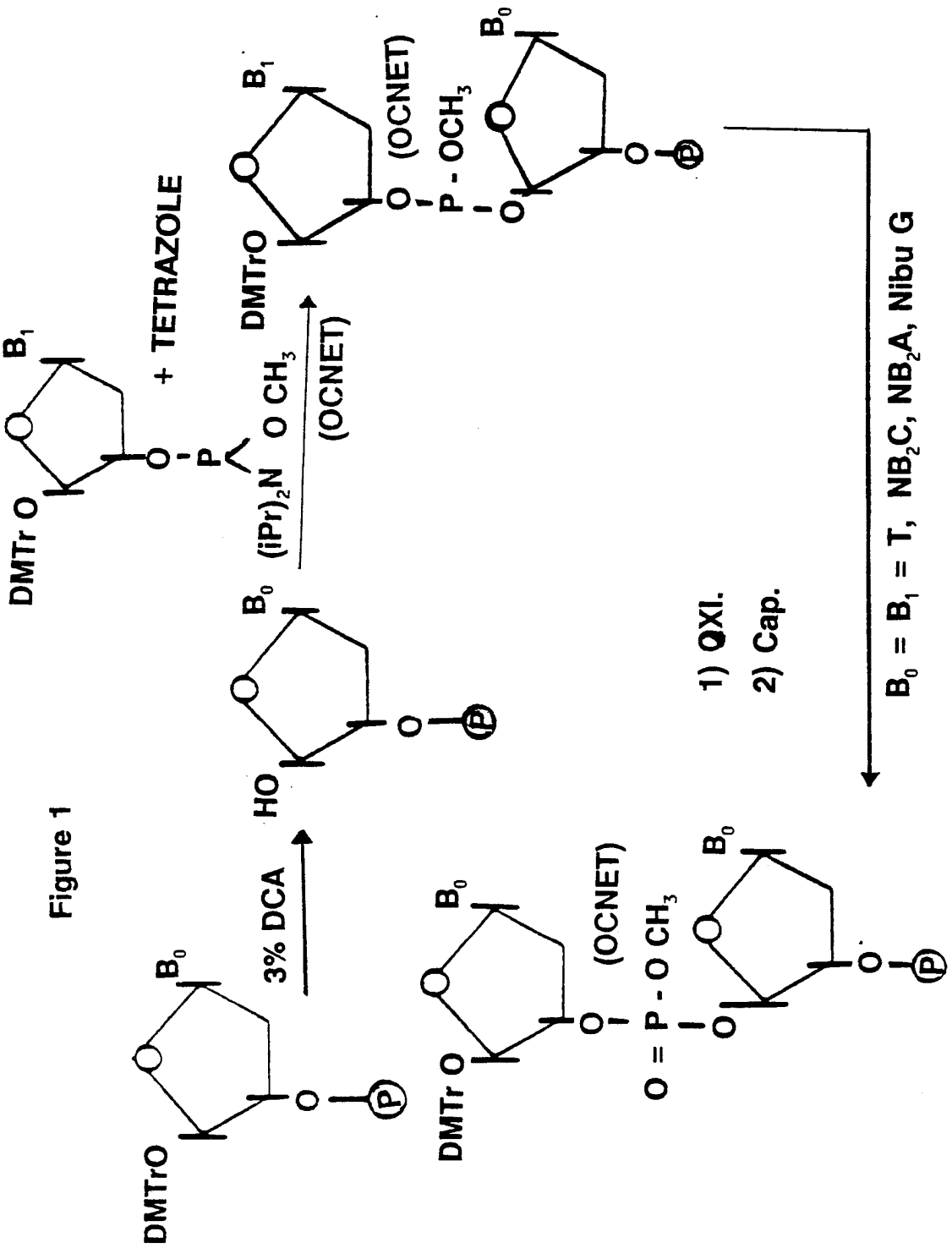
under suitable conditions so as to inhibit the replication of the virus.

20. A method of treating a subject with a disease which comprises administering to the subject an effective amount of the molecule of claim 1 and

a pharmaceutically acceptable carrier.

21. A method of claim 20, wherein administration comprises intravenous administration.

22. A method of claim 20, wherein administration comprises a time release implant.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P

Figure 1

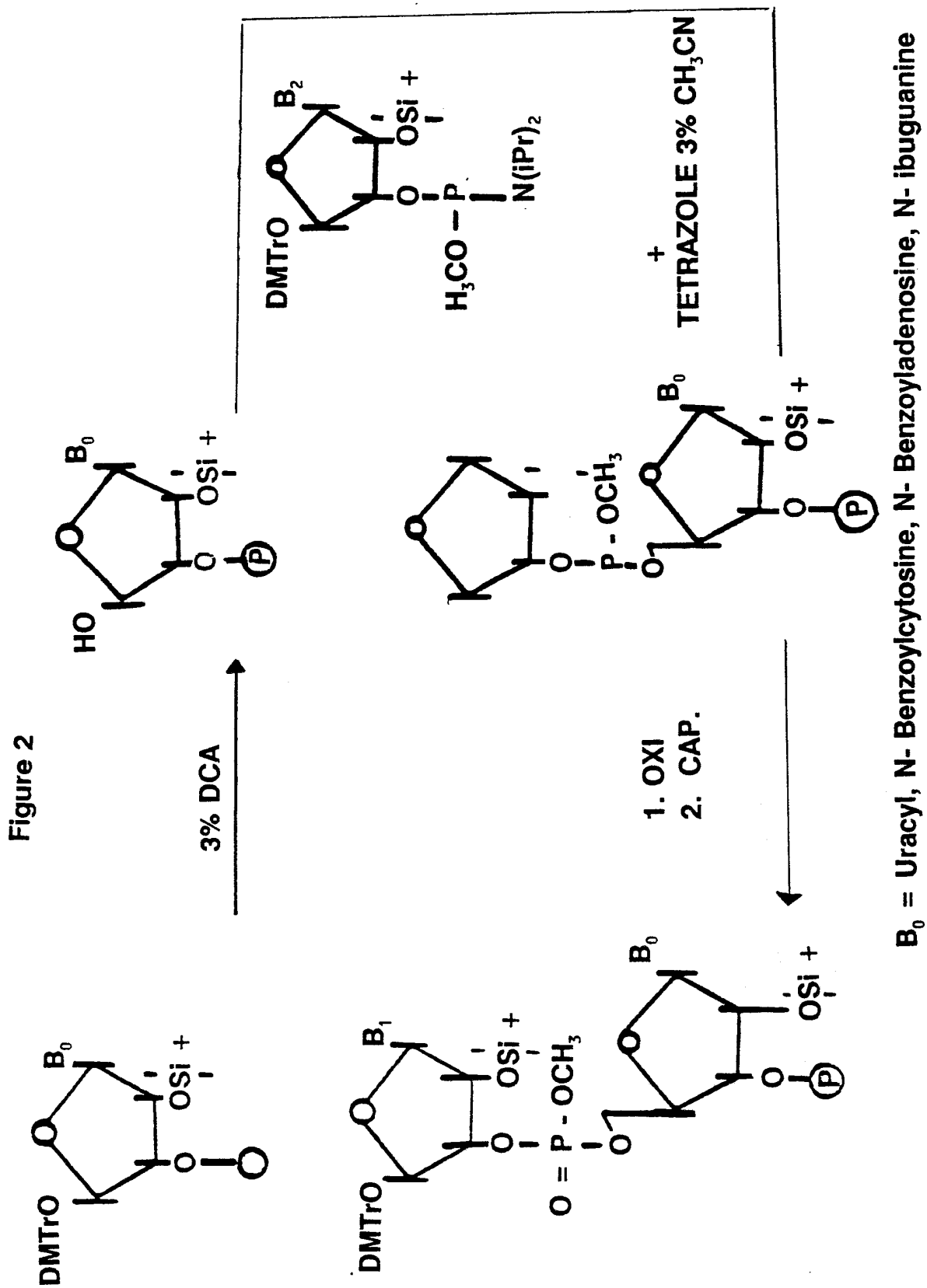

DMTrO
B0
OSi +
O
O
P
HO
3% DCA
DMTrO
B2
OSi +
O
P
H3CO
N(iPr)2
+
TETRAZOLE 3% CH3CN
B0
OSi +
O
P - OCH3
O
P
1. OXI
2. CAP.
DMTrO
B1
OSi +
O
O = P - OCH3
O
B0
OSi +
O
P
B0 = Uracyl, N- Benzoylcytosine, N- Benzyladenosine, N- ibuguanine

Figure 2

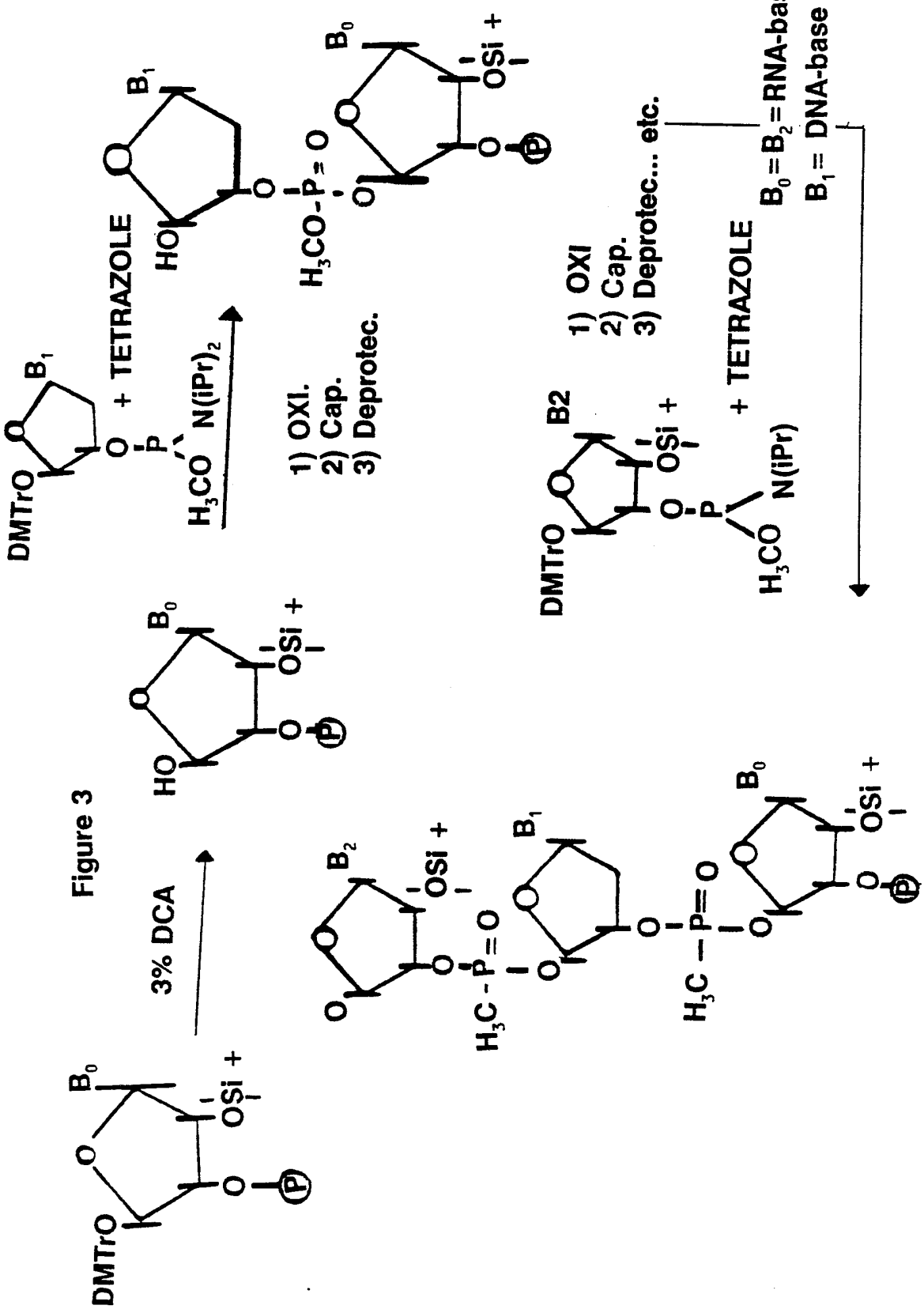

DMTrO
B1
O
P
H3CO N(iPr)2
+ TETRAZOLE
HO
H3CO-P= O
1) OXI.
2) Cap.
3) Deprotec.
B0
OSi +
3% DCA
B2
H3C-P=O
1) OXI
2) Cap.
3) Deprotec... etc.
B0 = B2 = RNA-base
B1 = DNA-base

Figure 3

Figure 4

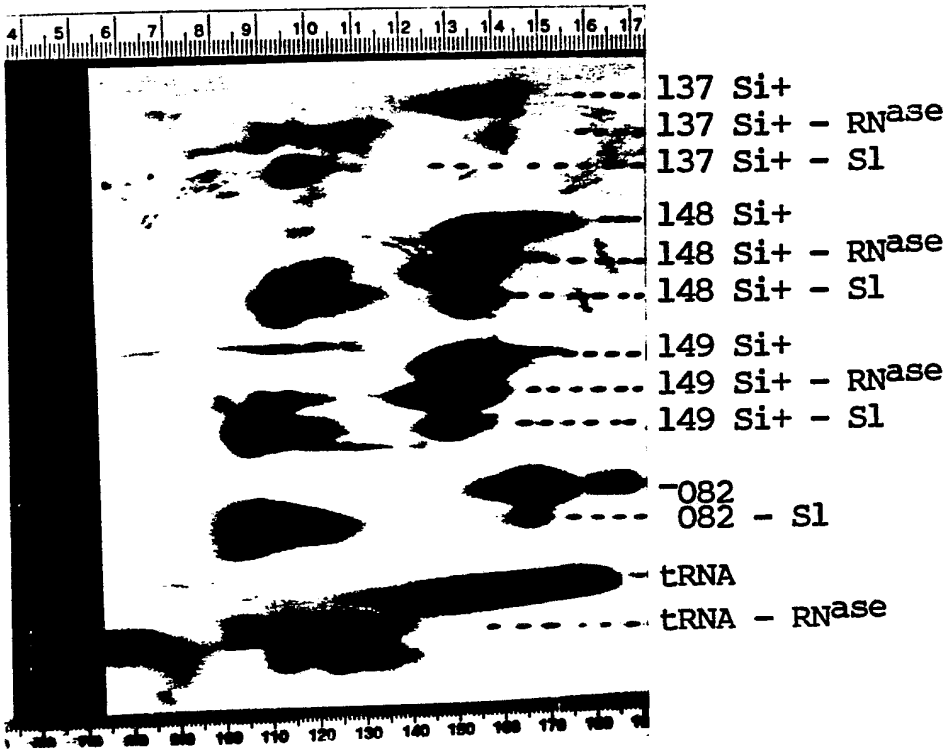
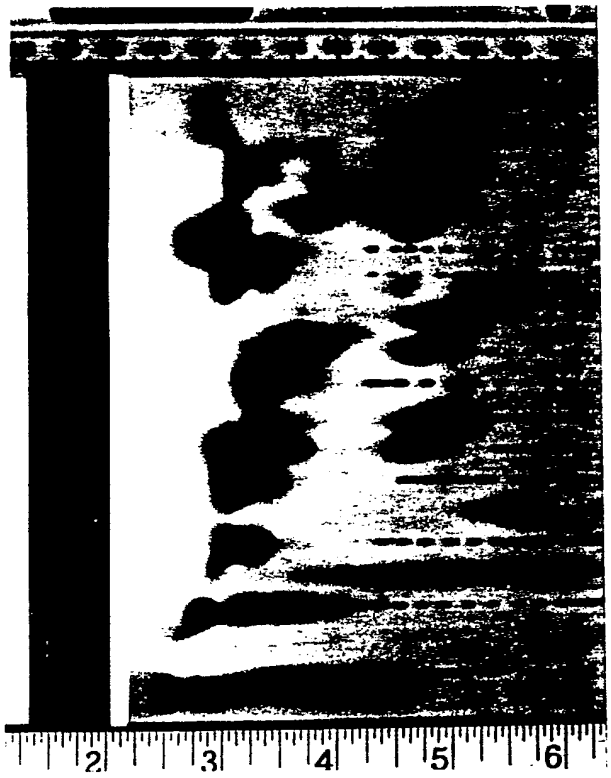
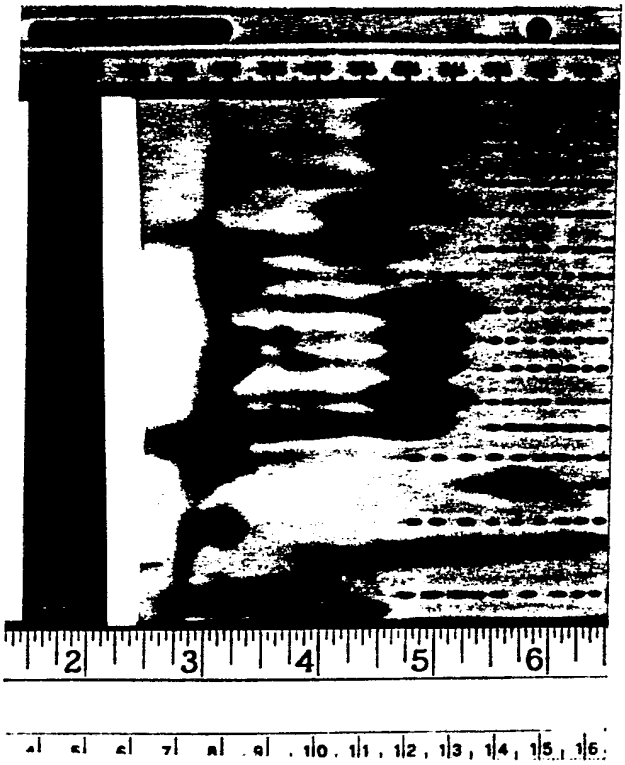

137 Si+
137 Si+ - RNase
137 Si+ - S1
148 Si+
148 Si+ - RNase
148 Si+ - S1
149 Si+
149 Si+ - RNase
149 Si+ - S1
082
082 - S1
tRNA
tRNA - RNase

Figure 5



136 Si+
136 Si+ - RNase
136 Si+ - S1
136 Si-
136 Si- - RNase
136 Si- - S1
148 Si-
148 Si- - RNase
148 Si- - S1
149 Si-
149 Si- - RNase
149 Si- - S1
082
082 - S1
tRNA
tRNA - RNase
tRNA - Fr. V

Figure 6


150 Si+
150 Si+ RNase
150 Si+ - S1
150 Si-
150 Si- - RNase
150 Si- - S1
150 Si+(s)
150 Si+(s) - RNase
150 Si+(s) - S1
150 Si-(s)
150 Si-(s) - RNase
150 Si-(s) - S1
082
082 - S1
tRNA
tRNA - RNase

INTERNATIONAL SEARCH REPOR.

International Application No PCT/US90/03486

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) 3

According to International Patent Classification (IPC) or to both National Classification and IPC

INT. Cl(5): C07H 21/04; C12Q 1/68

U.S. Cl: 536/27-29; 435/6

II. FIELDS SEARCHED

Minimum Documentation Searched 4

Classification System	Classification Symbols
U.S.	536/27,28,29
	435/6

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched 5

III. DOCUMENTS CONSIDERED TO BE RELEVANT 14

Category *	Citation of Document, 16 with indication, where appropriate, of the relevant passages 17	Relevant to Claim No. 18
Y	US, A, 4,806,463 (GOODCHILD ET AL) 21 February 1989, See Table II and claims.	1-17, 19
Y,P	US, A, 4,876,187 (DUCK ET AL) 24 October 1989, See entire document.	1-17, 19
Y	EP, A, 0,067,597 (BIOLOGICALS, INC.) 22 December 1982, See claims 1-21.	1-17, 19
Y	US, A, 4,689,320 (KAJI ET AL) 25 August 1987, See claims 1-22.	1-17, 19
Y	Proceedings of the National Academy of Science, Volume 86, No. 11, issued June 1989 (Washington, DC), MATSUKURA ET AL, "Regulation of Viral Expression of Human Immunodeficiency Virus in Vitro by an Antisense Phosphorothidate Oligodeoxynucleotide Against REV (DRT/JRS) in Chronically Infected Cells," See pages 4244-4248.	1-17, 19
Y	Proceedings of the National Academy of Sciences, Volume 85, issued February 1988, (Washington, DC), WICKSTRON ET AL, "Human Promyelacytic Leuremia HL-60 Cell Proliferation and C-MYC Protein Expre-sion are Inhibited by an Antisense Pentadecodeoxynucleotide (cont'd).	1-13, 19

* Special categories of cited documents: 15

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search 2	Date of Mailing of this International Search Report 2
06 AUGUST 1990	6 NOV 1990
International Searching Authority 1	**Signature of Authorized Officer 20**
ISA/US	Gary L. Kunz

Form PCT/ISA/210 (second sheet) (May 1986)
cdb:8/11/90

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category *	Citation of Document, [16] with indication, where appropriate, of the relevant passages [17]	Relevant to Claim No [18]
	Targeted Against C-MYC M RNA, See pages 1028-1032.	

FURTHER INFORMATION CONTINUED FROM THE SECOND SHEET

V. ☐ OBSERVATIONS WHERE CERTAIN CLAIMS WERE FOUND UNSEARCHABLE [1]

This international search report has not been established in respect of certain claims under Article 17(2) (a) for the following reasons:

1. ☐ Claim numbers ... , because they relate to subject matter [1] not required to be searched by this Authority, namely:

2. ☐ Claim numbers ..... , because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out [1], specifically:

3. ☐ Claim numbers_____, because they are dependent claims not drafted in accordance with the second and third sentences of PCT Rule 6.4(a).

VI. ☒ OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING [2]

This International Searching Authority found multiple inventions in this international application as follows:

Group I, claims 1-17 and 19, drawn to polynucleotide and method of inhibiting viral replication.
Group II, claim 18, drawn to a method of inhibiting Tumors.
Group III, claims 20-22, drawn to a method of inhibiting disease.

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims of the international application.

2. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims of the international application for which fees were paid, specifically claims:

3. ☒ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claim numbers:

Claims 1-17 and 19

4. ☐ As all searchable claims could be searched without effort justifying an additional fee, the International Searching Authority did not invite payment of any additional fee.

Remark on Protest

☐ The additional search fees were accompanied by applicant's protest.

☐ No protest accompanied the payment of additional search fees.

REASONS FOR HOLDING LACK OF UNITY OF INVENTION

This application contains the following inventions or groups of inventions which are not so linked as to form a single inventive concept. In order for all inventions to be examined, the appropriate additional examination fees must be paid.

Group I, claims 1 - 17 and 19, drawn to an enzyme-resistant polynucleotide and the method of using this polynucleotide to inhibit viral replication.

Group II, claim 18, drawn to a method of inhibiting tumor growth with this same polynucleotide.

Group III, claims 20 - 22, drawn to a method of treating "disease" using the claimed polynucleotides.

The inventions listed as Groups I, II, and III do not meet the requirements for Unity of Invention for the following reasons:

Groups I - III include not only polynucleotides resistant to enzymes but also three different method of using these polynucleotides. Unity of invention permits only one method of use of one product.

During a telephonic requirement for election, on August 7, 1990, applicant's representative, Ms. Sarah Adriano, elected the invention of Group I for examination. No additional examination fees were authorized and only one invention was elected.

Applicant stands advised that there is no right to protest the holding of lack of unity of invention for any group of invention(s) for which no additional examination fees has been paid. Any protest to the holding of lack of unity of invention or the amount of the additional fee required must be filed no later than one month from the date of this letter.

Any inquiry concerning this communication should be directed to Examiner Gary Kunz at telephone number 703-557-3517.